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In vivo transient rise in plasma free fatty acids alters the functional properties of α-fetoprotein

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Previous in vitro studies have shown that unsaturated fatty acids (UFA) induce conformational changes in rodent and human α-fetoprotein (AFP). To determine whether such changes in the binding and immunological properties of rat AFP also occur in vivo, plasma free fatty acid (FFA) concentrations were increased in young male rats (15, 21 and 28 days old) by acute i.v. injection of heparin (200 IU/kg). Plasma estrogens (estrone and estradiol) did not change after injection of heparin. There was a large increase in plasma FFA 10–20 min post-heparin injection, with a return to normal 60 min later. This transient rise in FFA plasma was associated with a 50% drop ($P < 0.001$) in the binding of estradiol to rat AFP of 15-, 21- and 28-day-old rats by reducing the number of binding sites ($P < 0.001$), leaving the affinity constant (K_a) unchanged. FFA extracts from post-heparin plasma induced similar changes in estradiol binding to purified rat AFP. The rise in plasma FFA induced a loss of AFP immunoreactivity, in 21- ($P < 0.001$) and 28-day-old rats ($P < 0.001$), but not in 15-day-old rats. This age-dependent response correlated with the FFA/AFP molar ratio (38 in 15-day-old rats, 388 in 21-day-old rats, and 5600 in 28-day-old rats). These results indicate that an in vivo rise in FFA induces rapid and reversible conformational changes in AFP which may modulate the endocrine and immune function of this oncofetal protein.

Introduction

There is now considerable evidence that free fatty acids (FFA) play a major role in the transfer of hormonal information. They induce subtle specific changes in the binding of hormones to several plasma proteins, including murine α-fetoprotein (AFP) [1–8], human sex-binding protein (SBP) [9], human corticosterone-binding globulin (CBG) [10], and thyroxine-binding globulin (TBG) [11,12].

Previous studies have established that FFA inhibit estrogen binding to purified rat and mouse AFP. This inhibition is dose dependent, varies with their degree of unsaturation and results from a FFA-induced conformational change in AFP that is reflected in alterations in the immunological behaviour of this protein [7,13]. AFP from different species bind FFA, especially polyunsaturated fatty acids, with high affinity [4,5, 14,15], and some studies indicate that the functional properties of this protein depend on its endogenous FFA content, which in turn depends on its exogenous FFA environment [6,8]. These in vitro results led us to determine whether a single transient change in the plasma FFA concentration in vivo could affect the functional properties of the serum steroid binding protein, rat AFP.

To induce lipolysis in young male rats we used heparin, which is known to release hepatic lipase (HL) and lipoprotein lipase (LPL) from endothelium cells into the blood stream and to cause an increase in plasma free fatty acids [16].

In this study we have examined the influence of heparin-induced changes in plasma FFA on the physicochemical properties of AFP during two developmental periods of the rat, one in which the plasma concentration of AFP is high (15-day-old rats), and the second when the AFP concentration is lower (21-day-old and 28-day-old rats) [6].

The results indicate that the transient increase in plasma FFA induced by heparin causes significant but

Abbreviations: UFA, unsaturated fatty acids; AFP, α-fetoprotein; FA, fatty acids; FFA, free fatty acids; SBP, sex-binding protein; CBG, corticosterone-binding globulin; TBG, thyroxine-binding globulin; HL, hepatic lipase; LPL, lipoprotein lipase; RIA, radioimmunoassay.

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reversible conformational changes in the structure of AFP, which are reflected in altered binding properties and immunoreactivity.

Materials and Methods

Reagents

[6,7-^{3}H]Estradiol (55 Ci/mmol) and [2,4,6,7-^{3}H]estrone (98 Ci/mmol), purchased from the Amersham International (Amersham, UK) were 98–99% pure. They were regularly tested to ensure that level of purity.

Unlabelled steroids estradiol (E2) and estrone (E1) and standard saturated and unsaturated free fatty acids were purchased from Sigma (St. Louis, MO). Heparin (200 IU/ml) was from Choay, Paris, France.

Animals and plasma preparation

Male Wistar rats (Charles River 76419 Saint-Aubin-les-Elbeuf, France), 15 days old weighing 36 ± 5 g and 21 days old weighing 55 ± 11 g, were kept in a controlled environment (20–22°C, light period 08.00–16.00). The 21-day-old rats had free access to standard diet pellets and tap water and the mid-suckling rats (15 days) were kept with their mothers until the experiment.

Groups of 15 rats of each age were injected intravenously with heparin (200 IU/kg in 0.5 ml 0.9% NaCl) via the tail vein under light ether anaesthesia.

Control rats were given 0.5 ml 0.9% NaCl. All experiments were performed between 9 and 12 h. Rats were bled 10, 20 and 60 min after injection; the blood was collected in glass tubes containing trisodium citrate (1.26 ml) on ice and immediately centrifuged at 3000 × *g* for 20 min. The plasma was stored at −20°C until analysis.

Extraction of steroids and FFA

Unlabelled heptadecanoic acid, [^{3}H]E1 and [^{3}H]E2 tracers were added to plasma samples (0.5 ml) as internal standard, each sample was extracted three times with organic solvent (ethyl acetate/cyclohexane, v/v) and the aqueous phase was removed by freezing (−20°C). The organic extracts were pooled, evaporated to dryness, taken up in 1 ml of chromatography solvent (benzene/ethanol, 95:5, v/v) (solvent I) and applied to Sephadex LH20 microcolumn (0.5 × 6 cm) equilibrated with benzene/ethanol (95:5, v/v). Free fatty acids were first eluted with 2.9 ml of solvent I. Estrone (E1) was then eluted with 3.5 ml of solvent I followed by 1 ml of solvent II (benzene/ethanol, 90:10, v/v). The fractions were evaporated to dryness and dissolved in benzene (1 ml). The yields from the extraction and purification steps were about 80%, as measured by the radioactive E1 and E2 standards, and by the unlabeled 17:0 standard FFA.

FFA analysis

The FFA fraction was evaporated to dryness and methylated in boron trifluoride/methanol (Merck). The methylated fatty acids were chromatographed on a Packard chromatograph, model 439 (Chrompack, Holland-Packard), using a capillary column (WCOT fused silica CP-sil-8CB 25 m × 0.32 mm). The column temperature was 151°C for the first 5 min, increasing thereafter by 3°C/min to 239°C, and by 10°C/min to 290°C. The injector temperature was 260°C and the detector temperature was 280°C.

The response coefficients and concentrations of FFA were determined with a recording chromatography data processor (Chromatopac CR1AB, Packard model 604). The peak area ratios of each FFA from plasma extracts and *n*-heptadecanoic acid (17:0) were compared to the peak area ratios of each FFA standard and 17:0. The total FFA concentration was obtained by summing all methylated derivatives on chromatography.

Blanks from all the buffers and solvents were run in parallel; no interfering exogenous contaminant was observed.

Immunoassay (RIA) of estrone and estradiol

The Sephadex LH20-purified sample of E1 was quantified by radioimmunoassay using rabbit anti-E1 6-thyroglobulin serum (Miles, Yeda Ltd, Israel). Estradiol (E2) was assayed in plasma by the Delfia method (Pharmacia). At least two measurements were made for each serum sample. The detection limit for estrogens was 18 pM. All antibodies were 100% specific for the hormones measured.

The blanks obtained by running the solvents through the whole extraction procedure were tested by radioimmunoassay, they produce no interference with the specific antibody.

Radioactivity was determined on samples, dissolved in 4 ml PCSII (Amersham) and counted in an Packard 1500 liquid scintillation spectrometer with the internal standard for technique quenching evaluation.

Serological methods

Purified rat AFP and monospecific rat AFP antibodies were obtained as previously described [2,7]. AFP was quantified by the Laurell rocket electroimmunodiffusion technique [17]. The detection limit of AFP was 0.015 μM.

Crossed immunoelectrophoresis was performed by the method of Laurell as modified by Clark and Freeman [18].

Dimension 1. Plasma samples (5 μl) were electrophoresed in 1% agarose gels at 8 V/cm using Tris-barbital lactic acid electrode buffer (pH 8.6) at 14°C.

Dimension 2. It was run overnight, at right angles to the first separation with the same buffer at 14°C at 2

V/cm into 1% agarose containing 0.5% monospecific anti-rat AFP antibody.

The gels were dried and stained with Coomassie blue.

Binding studies

The binding of [^{3}H]estradiol to rat plasma proteins was measured by the batchwise gel equilibration technique of Pearlman and Crépy [19] in phosphate buffer (H_2KPO_4/HNa_2PO_4, M/15, pH 7.4 at 21°C). The combining affinity indices or 'C values' (l/g) measure the ability of a mixture of proteins to bind a small molecule and is given by the expression: $C = (B/U)(1/P)$ where B, U and P are the concentrations of bound ligand (nM), unbound ligand (nM) and proteins (g/l), respectively [20].

Scatchard plots [21] were used to determine the affinity constant (K_a) and the apparent concentration of binding sites (nM). A series of assays with fixed amounts of plasma (2 μl for 15-day-old rats, 5 μl for 21-day-old rats) or purified rat AFP (3 μg = 29 nM) and [^{3}H]E2 (100000 cpm = 1.8 nM), plus increasing quantities of unlabelled E2 (0.9 to 184 nM) were performed.

Competition studies on the plasma free fatty acids fraction from controls or heparin-treated rats were carried out with fixed amounts of pure rat AFP (29 nM) and [^{3}H]E2 (100000 cpm = 1.8 nM), plus increasing quantities of organic solvent FFA extract from 21-day-old rats plasma at 10 min (♯ 1.5 mM) and 60 min (♯ 0.7 mM) post-injection.

Protein determination

Protein concentrations were assayed as described by Lowry et al. [22], with rat serum albumin as standard.

Statistical analysis

All data are reported as means ± S.E. Student's t-test was used to compare means. Results were considered significant when the probabilities were: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

Results

Plasma free fatty acid concentrations

The plasma free fatty acid concentrations of control 15- and 21-day-old rats were similar at all times after saline injection (Fig. 1), and were not significantly different from those of uninjected rats (data not shown).

The plasma FFA concentration of mid-suckling rats (15 days) increased significantly 10 min ($P < 0.001$), 20 min ($P < 0.01$) and 60 min ($P < 0.05$) after heparin injection.

The plasma FFA concentration of weaning rats (21 days) increased significantly only 10 min ($P < 0.001$) and 20 min ($P < 0.01$) after heparin injection. The increases occurred equally in all FFA classes, saturated and unsaturated (data not shown).

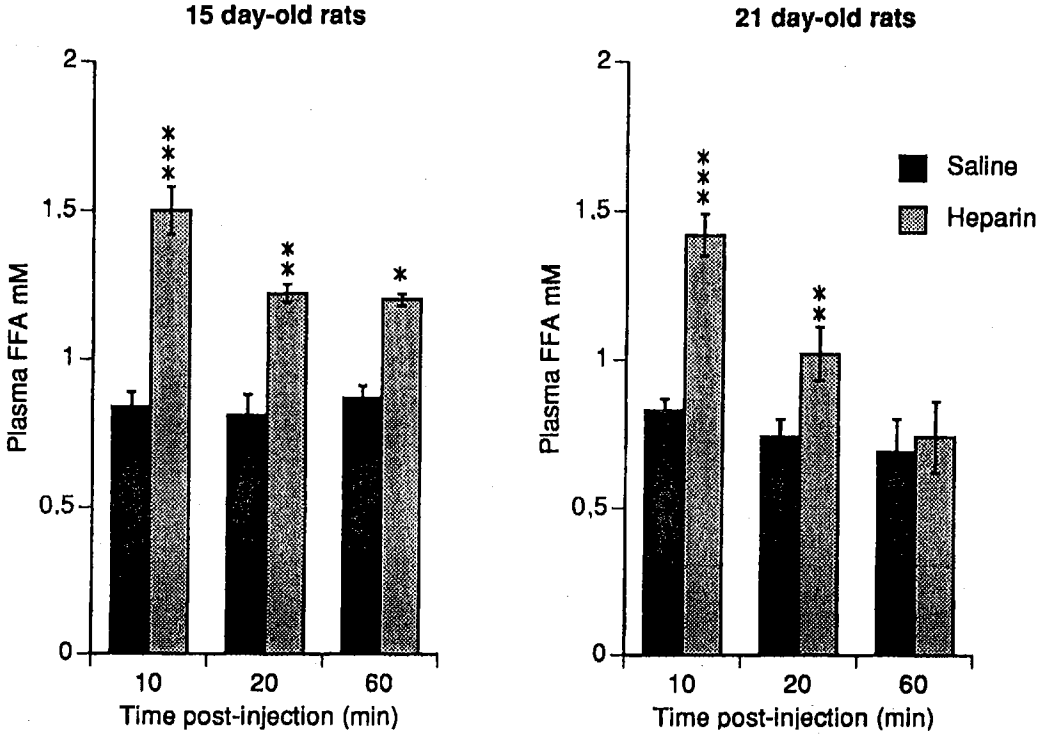

Fig. 1. Free fatty acid concentrations in the plasma of saline-injected control (■) and heparin-treated (□) rats. Values are means ± S.E. for 15 rats. Control and treated rats were compared using Student's t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

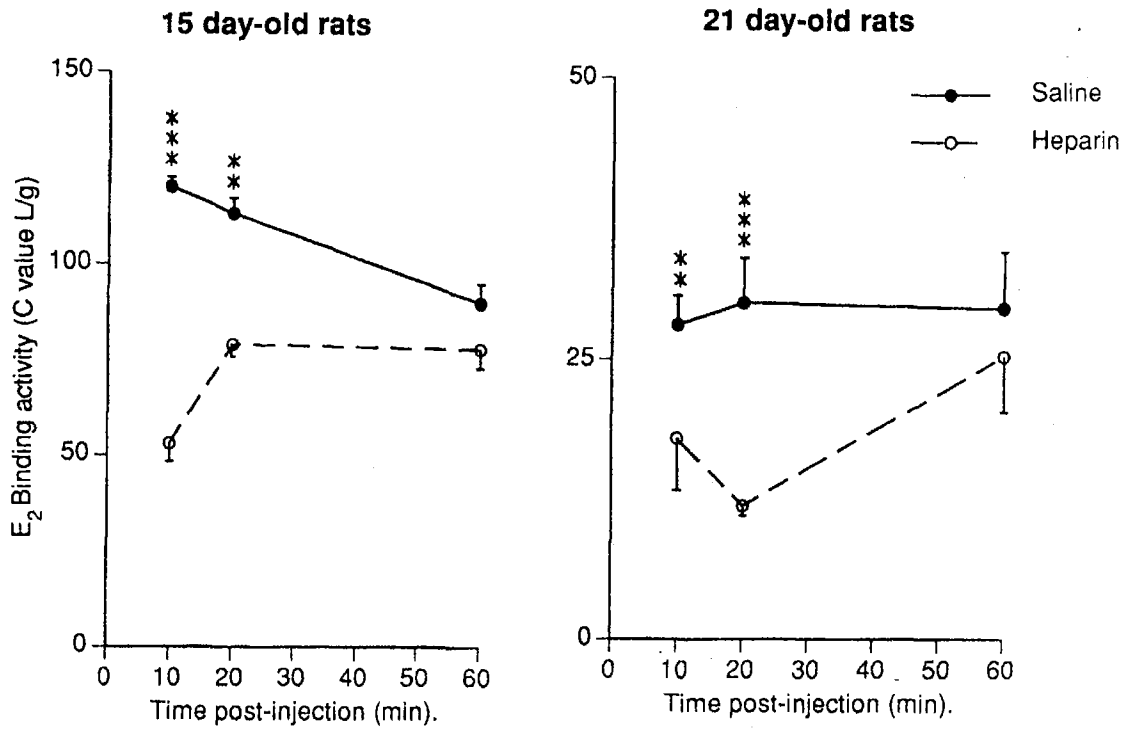

Fig. 2. Binding activities of plasma AFP (C values, l/g) following injection of 15- and 21-day-old rats with saline (•) or heparin (○). Estradiol binding was determined at 21°C by batch-wise gel equilibrium dialysis. The incubation mixture contained 2 μl (15-day-old rats = 42 mg protein/ml) or 5 μl (21-day-old rats = 55 mg protein/ml) plasma and [^{3}H]E2 (100000 cpm) in H_2KPO_4/Na_2HPO_4 buffer (M/15, pH 7.4). Values are means ± S.E. for 15 rats. Control and treated rats were compared using Student's t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. We have checked that the addition of heparin (50–200 IU/mg of plasma protein) did not influence in vitro the E2 binding to AFP in the plasma of 15- and 21-day old rats.

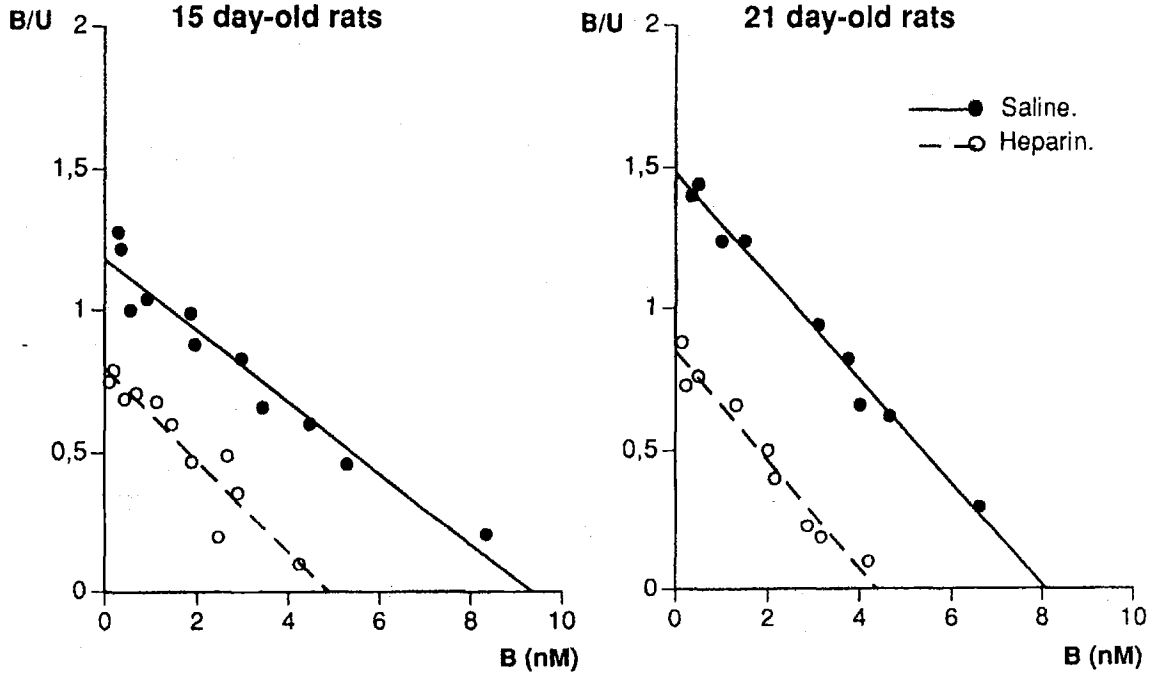

Fig. 3. Scatchard plots with Rosenthal correction for the interaction of [^{3}H]E2 binding with plasma AFP from control (•) and heparin-treated (○) 15- and 21-day-old rats. (A) 15-day-old rats. 2 μl of control or heparin-treated plasma taken 10 min post-injection was incubated with [^{3}H]E2 (100000 cpm) and 0.9–184 nM of E2, in H_2KPO_4/HNa_2PO_4 buffer (M/15, pH 7.4). (B) 21-day-old rats. 5 μl control or heparin-treated plasma taken 20 min post-injection was incubated as in A. B, bound steroid; U, unbound steroid. The E2 binding parameters K_a and nM are shown in Table I.

Plasma concentration of estrogens in heparin-treated rats

The plasma E2 and E1 concentrations of control rats were three times higher in the 15-day-old rats (3.24 ± 0.2 nM and 4.2 ± 0.19 nM) than in the 21-day-old rats (1.7 ± 0.2 nM and 1.19 ± 0.10 nM, respectively). Heparin treatment did not significantly alter the plasma E2 and E1 concentrations of either the 15-day-old (E2, 3.5 ± 0.04 nM; E1, 4.45 ± 0.13 nM) or the 21-day-old rats (E2, 1.72 ± 0.02 nM; E1, 1.13 ± 0.1 nM) at any time post-heparin injection.

Estradiol (E_2) binding to AFP

The E2 binding to control and heparin-treated rat plasma, expressed as C values (l/g), is shown in Fig 2. The C values for [^{3}H]E2 binding to AFP in post-heparin plasma from 15- and 21-day-old rats were both significantly lower 10 min ($P < 0.001$ and $P < 0.01$) and 20 min ($P < 0.01$ and $P < 0.001$) after heparin injection than those of control plasma. But the C values of E2 had returned to control values in heparin-injected rats by 60 min post-injection. These results indicate that the heparin-induced decrease in E2 binding to AFP is reversible and parallels the transient change in plasma FFA.

The association constant (K_a) and number of binding sites (nM) for E2 binding were determined by Scatchard analysis. Fig. 3 shows the Scatchard plot of control and post-heparin plasma from 15- and 21-day-old rats at 10 min and 20 min respectively, and Table I summarizes the E2 binding parameters at different times.

The decrease in C values for [^{3}H]E2 binding to AFP caused by heparin-induced lipolysis seems to be mainly due to a significant decrease in the number of binding sites ($P < 0.001$), while the affinity constant (K_a) was not affected.

The number of E2 binding sites (nM) in control and heparin-treated rats were the same at 60 min post-injection, in agreement with the observation that the C

TABLE I

Association constant (K_a) and number of binding sites (nM) from Scatchard analysis of [^{3}H]E2 binding to control and heparin-treated plasma from 15- and 21-day-old rats

		K_a (10^8 M^{-1})	nM
15-day-old rats			
control	10 min	1.3 ± 0.1	8.35 ± 0.35
	60 min	1.3 ± 0.2	8.00 ± 0.45
heparin	10 min	1.3 ± 0.2	5.22 ± 0.03 ***
	60 min	1.6 ± 0.4	6.65 ± 0.05 **
21-day-old rats			
control	10 min	1.5 ± 0.3	8.50 ± 0.20
	20 min	1.4 ± 0.4	8.35 ± 0.15
	60 min	1.5 ± 0.2	8.70 ± 0.50
heparin	10 min	1.6 ± 0.2	5.35 ± 0.15 **
	20 min	1.5 ± 0.3	4.38 ± 0.12 ***
	60 min	1.5 ± 0.2	7.75 ± 0.25

** $P < 0.01$.
*** $P < 0.001$.

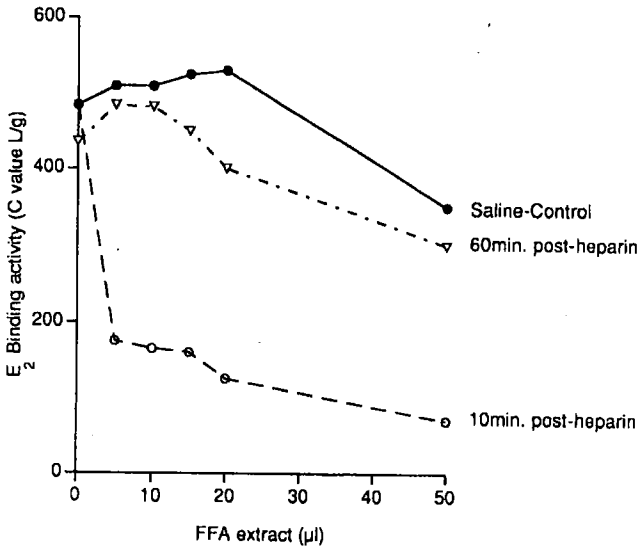

Fig. 4. Effect of increasing FFA extracted from saline or heparin-treated plasma on E2 binding activities of pure rat AFP. 3 µg (29 nM) rat AFP was incubated with [^{3}H]E2 (100000 cpm) in the presence of FFA extracts of plasma from 21-day-old rats, taken 10 min after saline (0.75 µmol/ml) (●), or heparin post-injection (1.47 µmol/ml) (○) or FFA extracts of plasma taken 60 min after heparin injection (0.65 µmol/ml) (△).

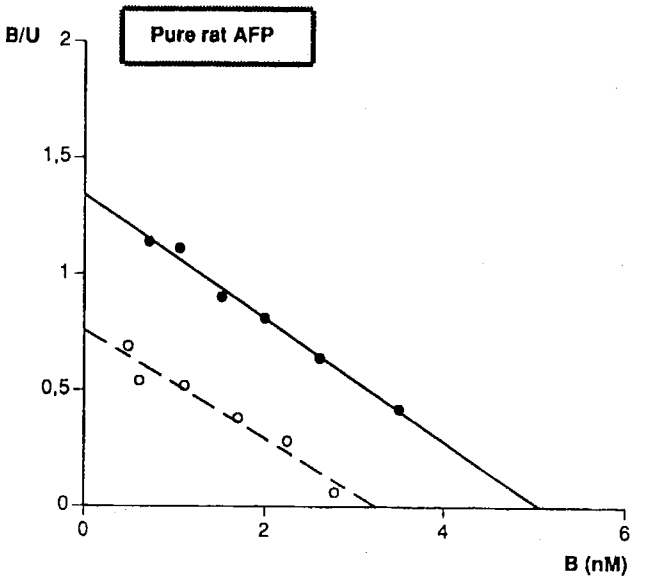

Fig. 5. Scatchard analysis of [^{3}H]E2 binding to purified rat AFP with (○) or without (●) 5 µl (7 nmol) FFA extracted from the plasma of 21-day-old rats taken 10 min after heparin injection. 3 µg (29 nM) purified rat AFP was incubated for 1 h at 21°C with [^{3}H]E2 (100000 cpm) and E2 (0.9–184 nM). Each point is the mean of duplicate assays. B, bound estradiol; U, unbound estradiol. Without 5 µl FFA extract, $K_a = 2.60 \cdot 10^8$ M^{-1} and nM = 5. With 5 µl FFA extract, $K_a = 2.34 \cdot 10^8$ M^{-1} and nM = 3.

values both heparin-treated and control 21-day-old rats were not statistically different at this time. Thus, the decrease in E2 binding sites observed at 10 min and 20 min was reversible at 60 min and correlated with the FFA level.

The E2 binding (C values) to AFP in the plasma of 15- and 21-day-old control rats was not disturbed in vitro by the addition of heparin (50 IU to 200 IU/mg of plasma protein) (see legend of Fig. 2).

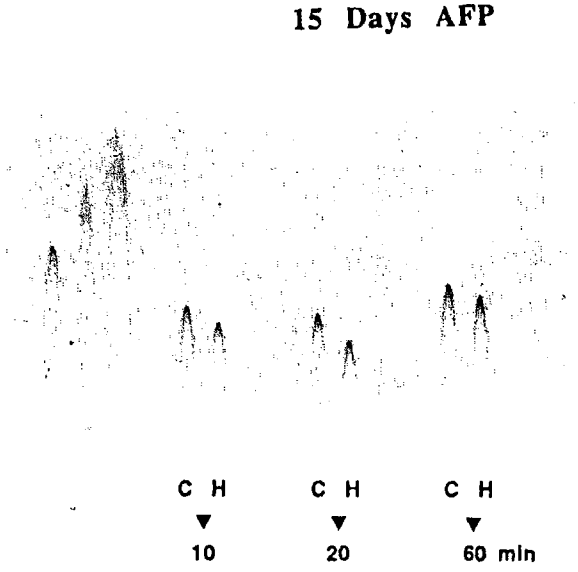

Fig. 6. Immunoquantification of AFP from 15- and 21-day-old rat plasma, taken 10, 20 and 60 min after saline or heparin injection. 5 μl control (C) or heparin-treated plasma (H) were electrophoresed overnight in 1% agarose containing 0.5% anti-rat AFP antibody at 8 V/cm using Tris-barbital lactic acid electrode buffer (pH 8.6) at 14°C. The first four wells contained pure rat AFP (10, 13.3, 20 and 26.6 μg/ml, respectively).

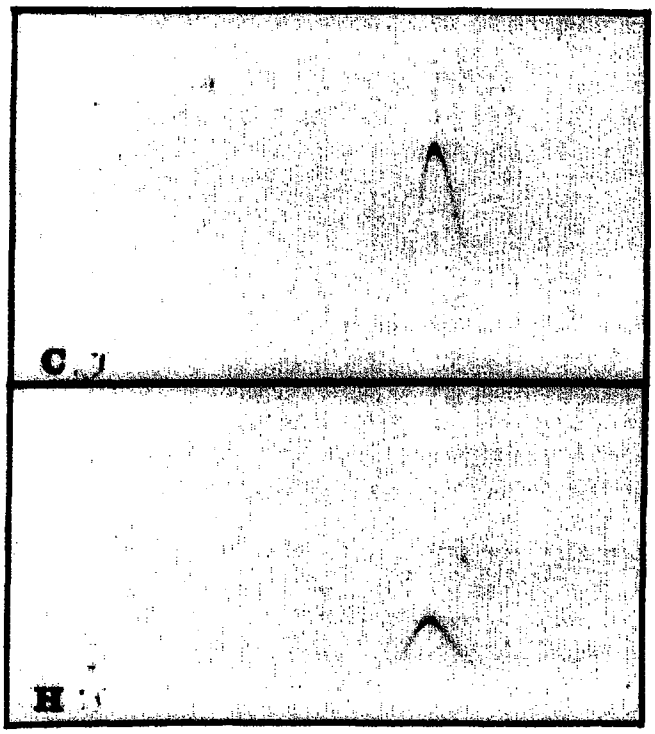

Fig. 7. Crossed immunoelectrophoresis of AFP in control (C) and heparin-treated plasma (H), taken 20 min after heparin injection. First dimension gel contained 5 μl control (C) or heparin-treated plasma (H). Second dimension gel contained 0.5% anti-rat AFP antibody. The gels were dried and stained with Coomassie blue.

Effect of FFA extracts of post-heparin plasma on estradiol binding to purified rat AFP in vitro

FFA extracts (1.47 μmol/ml) of plasma taken from 21-day-old rats 10 min after heparin injection produced a dose-dependent inhibition of E2 binding to rat AFP (C values, 1/g) (Fig. 4). The inhibitory potency (IC_{50} values) for E2 binding was 20-fold higher for FFA extracts of plasma taken 10 min than those taken 60 min after heparin injection.

Scatchard analysis of E2 binding to pure rat AFP in the presence of 5 μl FFA extracts (7 nmol) of plasma from 21-day-old rats taken 10 min after heparin treatment (Fig. 5) indicated that the FFA extracts inhibited E2 binding to rat AFP, as in vivo with a decrease in the concentration of E2 binding sites (nM) and no significant change in K_a.

Immunoquantification of AFP

The concentrations of AFP in the plasma of control and heparin-treated rats were assessed by rocket immunoelectrophoresis using monospecific anti-AFP antibodies. Lipolysis appeared to have no statistically significant effect on the immunological properties of AFP from mid-suckling 15-day-old rats (Fig. 6 and Table II). By contrast there was a significant decrease

TABLE II

	FFA (μM)	Apparent AFP concentration (μM)	'C values' (l/g)	Apparent FFA/AFP molar ratio
Control rats				
15 days	840 ± 50	22 ± 1.28	108.0 ± 9.0	38
21 days	750 ± 70	1.93 ± 0.07	30.0 ± 4.0	388
28 days	340 ± 50	0.06 ± 0.01	0.9 ± 0.1	5600
20 min heparined rats				
15 days	1500 ± 80	22 ± 2.14	53.0 ± 4.0	68
21 days	1020 ± 90	0.77 ± 0.07	12.0 ± 0.7	1325
28 days	640 ± 40	0.014 ± 0.01	0.5 ± 0.1	45700

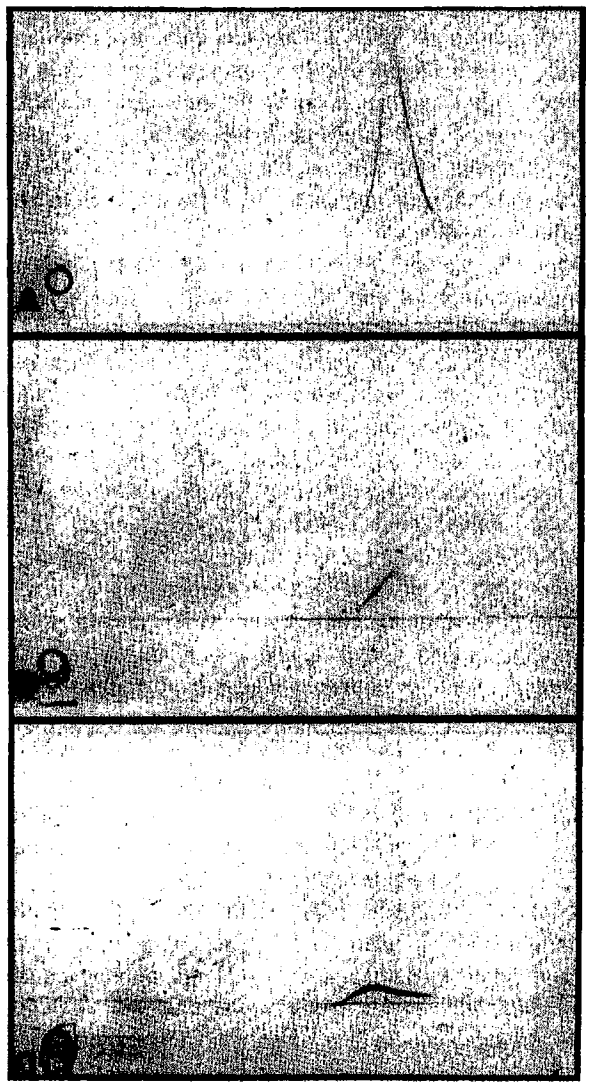

Fig. 8. Effect of FFA on purified AFP immunoreactivity in vitro. Crossed immunoelectrophoresis was carried out on AFP alone (A), AFP incubated with FFA extracted from heparin-treated rat plasma (B) and AFP incubated with a mixture of FFA standards (C). Antiserum: rabbit anti-rat AFP (0.3%). (A) Purified rat AFP (1.8 pmol). (B) Purified rat AFP (1.8 pmol) incubated overnight at 4°C with 104 nmol FFA extracted from the plasma of 21-day-old rats 10 min after heparin injection (FFA extract composition: 33% saturated FA, 14% monounsaturated FA, 53% polyunsaturated FA). (C) Purified rat AFP (1.8 pmol) incubated overnight at 4°C with 50 nmol standard FFA mixture (33% saturated FA, 14% monounsaturated FA, 53% polyunsaturated FA).

($P < 0.001$) in the amount of AFP in the plasma of 21-day-old rats (Fig. 6), especially 20 min after heparin injection (2 μM in control plasma and 0.77 μM in heparin-treated plasma). The amounts of AFP in the plasma of control and heparin-treated rats were essentially the same 60 min post-injection (Fig. 6) (2 μM in control and heparin-treated rats).

Fig. 7 shows the crossed immunoelectrophoresis patterns for AFP in the plasma from 21-day-old rats taken 20 min after saline or heparin injection. The immunorecognition of AFP by anti-AFP antibodies is clearly decreased after heparin-induced lipolysis, without any significant change in the mobility of AFP. Similar results were obtained with plasma AFP from 28-day-old rats, especially 10 min and 20 min after heparin injection (data not shown).

The immunoreactivity of AFP was unaffected by the circulating concentration of heparin.

The immunological behaviour of purified AFP loaded with FFA extracted from heparin-treated plasma from 21-day-old rats or FFA standard mixture (with a composition close to that of the extract) was analysed to determine whether the decrease in immunoreactivity of heparin-treated plasma AFP was due to a FFA-induced conformational change in AFP.

Fig. 8 shows that both AFP loaded with FFA plasma extracts (Fig. 8b) and AFP loaded with a FFA standard mixture (Fig. 8c) were poorly recognized by anti-AFP antibodies directed against native AFP (i.e., AFP not loaded with FFA). Increasing the FFA standard mixture concentration also decrease the immunoreactivity of purified AFP in a dose-dependent manner (data not shown). Previous studies [7] have shown that adding FFA to purified AFP without prior incubation does not disturb the immunodetection of AFP.

Discussion

These results indicate that the transient rise in plasma FFA which occurs in vivo after heparin-induced lipolysis alters the functional properties of the steroid carrier protein, rat AFP. There was a clear relationship

between the rise in plasma FFA and conformational changes in AFP.

The significant increases (30 to 80%) in plasma FFA, especially 10 min and 20 min after heparin injection, without changes in plasma estrogen concentration, lead to a significant decrease (50%) in the estradiol-binding properties of rat AFP in 15-day, 21-day and 28-day-old rats. The E2-binding activities of heparin-treated rat AFP and saline-treated rat AFP were not significantly different 60 min after injection, which correlates well with transient nature of the rise in plasma FFA.

Thus, the inhibition of E2 binding to rat plasma AFP, which parallels the rise in plasma FFA concentration induced by heparin, is rapid and reversible.

The Scatchard analysis indicates that the apparent number of E2 binding sites on rat AFP were decreased by 50%, but the affinity constant (K_a) was unaffected in high-FFA plasma taken 10 and 20 min. post-heparin. The involvement of FFA in these changes was confirmed by the experiments showing that FFA extracts from the plasma of heparin-treated 21-day-old rats inhibited E2 binding to purified rat AFP in a dose-dependent manner. But the quantities required to produced 70% inhibition of E2 binding to purified rat AFP were 2-fold lower than with AFP in post-heparin plasma, suggesting that other proteins may interfere and/or bind FFA, as has been shown in vitro for human SBP, CBG, albumin, and lipoproteins [9,10,23–25].

FFA extracts markedly (70%) decrease E2 binding to pure rat AFP and mimic the in vivo situation, with Scatchard plots showing a 40% decrease in the number of E2 binding sites.

Previous in vitro studies using unsaturated FFA standards [7], suggest that the increased FFA saturates sites that do not affect the K_a of E2 binding sites (non-competitive binding).

The immunological results provide additional evidence for FFA-mediated conformational changes in AFP. The rise in FFA induces loss of immunoreactivity, especially in 21-day and 28-day-old rats. Polyclonal antibodies raised to native rat AFP (N-AFP) recognized fewer epitopes on heparin-induced lipolysis plasma AFP (L-AFP) than on N-AFP. Our studies performed with purified AFP show that FFA extracted from the plasma of heparin treated rats and FFA standard mixture both give rise to poor immunodetection of AFP.

Mixing heparin or FFA (120 nmol) with N-AFP without any incubation did not disturb the immunodetection of N-AFP. Thus, as previous in vitro studies [7], the perturbation of immunoprecipitation is probably caused by FFA binding to AFP, with a conformational change in L-AFP causing rearrangement of surface antigen sites to bring about quantitative and qualitative alteration in the reaction with antibody. However, the immuno-reactivity of the AFP in the plasma of heparin-treated 15-day-old rats was unchanged. The difference between immunoquantification of AFP in the post-heparin plasma from 15 day and older rats, and especially the misdetection of AFP in 21 day and 28 day plasma may be correlated with the fact that the plasma of 21- and 28-day-old rats contains a high concentration of FFA (0.75 mM and 0.35 mM, respectively) and a low AFP concentration (1.93 μM and 0.06 μM, respectively) (Table II). Thus, the immunological results indicate that the effect of FFA on AFP conformational changes depends on the FFA/AFP molar ratio: this ratio is 38 in 15-day-old rats, and 388 and 5600 in 21- and 28-day-old rats, respectively (Table II). However, other factors, such as the presence of different isoforms of AFP could be involved [6]. The combination of forms found in the plasma of 21- and 28-day-old rats may be more sensitive to fatty acids than that of 15-day-old rats.

Thus, the present in vivo data confirm the physiological relevance of our in vitro finding that unsaturated FFA induce conformational changes in rat AFP. As lipase activity is localized within cell membranes, it is likely that the concentration of fatty acids at these locations is higher than in the general circulation [26], and these in turn could influence AFP function. High FFA levels occur during stress, fasting, diabetes, obesity, and non-thyroidal illness [27–29]; under these circumstances the FFA/AFP ratio can be as much as 10-times that of normal. These findings also suggest that the FFA-induced changes in AFP hydrophobicity and conformation could modify AFP uptake by cell membranes such as lymphocytes [30], and so facilitate its internalization by target cells.

One of the most striking findings is the reversible effect of the rise of FFA on the binding and immunological properties of AFP, indicating that AFP could adapt to, and interact with the environmental changes which occur during ontogenesis, oncogenesis and in pathological situations. Our data also indicate that endogenous FFA from membranes may modify the 3-dimensional structure of AFP, and perhaps other serum hormone carrier proteins, such as SBP, CBG, or TBG, contributing to the formation of transient forms (holoforms). These changes could underly the multiple functional potentiality of AFP in endocrine, cell growth, and immune processes.

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